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TNF-α as a potential mediator of cardiac dysfunction due to intracellular Ca^{2+}-overload

Ming Zhang[a], Yan-Jun Xu[a], Harjot K. Saini[a], Belma Turan[a], Peter P. Liu[b], Naranjan S. Dhalla[a,*]

[a] Department of Physiology, St. Boniface General Hospital Research Center, Institute of Cardiovascular Sciences, Faculty of Medicine University of Manitoba, Winnipeg, Canada
[b] Division of Cardiology, Heart and Stroke/Richard Lewar Centre of Excellence, University of Toronto, Toronto, Canada

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Abstract

TNF-α has been shown to be involved in cardiac dysfunction during ischemia/reperfusion injury; however, no information regarding the status of TNF-α production in myocardial injury due to intracellular Ca^{2+}-overload is available in the literature. The intracellular Ca^{2+}-overload was induced in the isolated rat hearts subjected to 5 min Ca^{2+}-depletion and 30 min Ca^{2+}-repletion (Ca^{2+}-paradox). The Ca^{2+}-paradox hearts exhibited a dramatic depression in left ventricular developed pressure, a marked elevation in left ventricular end diastolic pressure, and more than a 4-fold increase in TNF-α content. The ratio of cytosolic to homogenate nuclear factor-κB (NFκB) was decreased whereas the ratio of phospho-NFκB to total NFκB was increased in the Ca^{2+}-paradox hearts. All these changes due to Ca^{2+}-paradox were significantly attenuated upon treating the hearts with 100 μM pentoxifylline. These results suggest that activation of NFκB and increased production of TNF-α may play an important role in cardiac injury due to intracellular Ca^{2+}-overload.

Keywords: Pentoxifylline; Ca^{2+}-paradox; Tumor necrosis factor-α; Nuclear factor-κB; Cardiac dysfunction

Tumor necrosis factor-α (TNF-α) is produced and released from different types of cells such as lymphocytes, vascular cells, and cardiomyocytes [1]. TNF-α is known to dimerize the TNF receptors on the surface of cell membrane which trigger a cascade of signals that lead to altered cell phenotype, including survival and death signals [1]. This cytokine has also been reported to depress contractile function [2,3], provoke the induction of apoptosis in different settings [4,5], and is involved in cardiac remodeling due to myocardial infarction and mitral regurgitation [6–8]. Some studies have demonstrated increased formation and release of TNF-α in hearts subjected to ischemia/reperfusion (I/R) injury [4,9,10] and have suggested that this cytokine may be involved in the I/R-induced cardiac dysfunction [4,9,10]. It is pointed out that both oxidative stress and Ca^{2+}-overload are considered to be the major mechanisms for I/R-induced cardiac injury [11,12]. While oxidative stress has been demonstrated to trigger the production and release of TNF-α [7], the role of Ca^{2+}-overload in promoting the formation of this cytokine has not been investigated. Because Ca^{2+}-paradox heart has been regarded to form an excellent model for studying the effects of Ca^{2+}-overload at cellular level [13,14], this study was undertaken to examine if the formation of TNF-α is increased in hearts subjected to Ca^{2+}-paradox. Since pentoxifylline (PTXF), a phosphodiesterase inhibitor, is known to depress TNF-α synthesis in heart failure and cardiomyopathy [15,16], we tested the effects of

* Corresponding author. Fax: +1 204 233 6723.
E-mail address: nsdhalla@sbrc.ca (N.S. Dhalla).

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this agent on cardiac function and the levels of TNF-α in Ca^{2+}-paradox heart. Furthermore, in view of the role of nuclear factor-κB (NFκB) in the synthesis of TNF-α [17,18], the activated form of NFκB and redistribution of NFκB were also measured in this study.

Materials and methods

Perfusion of the isolated rat hearts. Male Sprague–Dawley rat (280–350 g) hearts were isolated and perfused by the Langendorff technique at a constant flow rate of 10 ml/min. The perfusion medium, Krebs–Henseleit (K–H) buffer containing in mmol/L: 120 NaCl, 4.8 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose (pH 7.4), was maintained at 37 °C and gassed continuously with a mixture of 95% O_2 and 5% CO_2. The hearts were stimulated electrically at 300 beats/min by using Phipps and Bird stimulator (Richmond, VA). A water-filled elastic balloon was inserted into the left ventricle and the left ventricular end diastolic pressure (LVEDP) was adjusted at 9–10 mmHg at the beginning of the experiment. The left ventricular developed pressure (LVDP), LVEDP, rate of pressure development ($+dP/dt$), and rate of pressure decay ($-dP/dt$) were measured using AcqKnowledge 3.5 for Windows 3.0 (Biopac System, Goleta, CA). Data were recorded online through an analogue-digital interface (MP 100, Biopac System, Goleta, CA). All hearts were stabilized for a period of 20 min before use.

Experimental protocol. The Ca^{2+}-paradox was induced as in previous studies [13,14]. The hearts were divided into three groups. For the control group, the hearts were perfused with oxygenated K–H medium for 35 min. For the Ca^{2+}-paradox group, the hearts were perfused with Ca^{2+}-free medium for 5 min followed by 30 min perfusion with normal K–H buffer containing 1.25 mmol/L Ca^{2+}. For the PTXF treatment group, PTXF (100 μM) infusion was started 10 min before inducing Ca^{2+}-paradox and was carried out throughout the Ca^{2+}-depletion and Ca^{2+}-repletion periods. The selection of this concentration of PTXF was based on our observations showing maximal improvement of cardiac function in this experimental model. After assessment of the left ventricular function, the hearts were frozen in liquid N_2 and stored at −70 °C for biochemical analysis.

Measurement of TNF-α. Ventricular tissue was homogenized in 10 volumes of phosphate-buffered saline (PBS), which contained 1% Triton-100 (Sigma–Aldrich, Oakville, Ont., Canada) along with a protease inhibitor cocktail (Roche, Laval, Que., Canada) [10]. The homogenate was centrifuged at 2500*g* for 20 min at 4 °C. The supernatant was collected and the TNF-α level was measured using a sandwich ELISA kit for rat TNF-α with a 12.5 pg/ml detection limit (R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer's instructions. Absorbance of standards and samples was determined spectrophotometrically (SPECTRAmax PLUS^{384}, Molecular Devices, Sunnyvale, CA) at 450 nm. Results were calculated from the standard curve and were reported as pg/g protein.

Western blot for NFκB. Ventricular tissue (50 mg) was homogenized (Polytron PT 3000, Brinkmann Instruments, Mississauga, ON, Canada) on ice (at setting 8 for 2 × 30 s with 30 s interval in between) in 1 ml buffer A containing: 50 mmol/L Tris–HCl, 0.25 mol/L sucrose, 10 mmol/L EGTA, 4 mmol/L EDTA, and protease inhibitor cocktail, pH 7.5. The suspension was sonicated for 2 × 15 s with 30 s interval in between and centrifuged at 100,000*g* for 60 min in an ultracentrifuge (Model L70, Beckman Instruments, Fullerton, CA). The supernatant was collected and labelled as cytosolic fraction. The pellet was suspended in 1 ml buffer B (buffer A + 1% Triton X-100), incubated on ice for 60 min, and centrifuged at 100,000*g* for 60 min in an ultracentrifuge. This supernatant containing dissolved particulate protein was labelled as particulate fraction. Another piece of 50 mg ventricular tissue was suspended in buffer B, homogenized, and sonicated as above. The homogenate was incubated on ice for 60 min and centrifuged at 100,000*g* for 60 min in an ultracentrifuge. The supernatant thus obtained was labelled as homogenate fraction. This method for preparing tissue extract is the same as described elsewhere [19].

The immunoblotting analysis of total-NFκB and phosphorylated NFκB (phospho-NFκB) was performed by separation of 20 μg protein on a 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins separated by SDS–PAGE were electroblotted to polyvinylidene difluoride membrane (PVDF) by employing a transfer buffer containing 25 mmol/L Tris–HCl, 192 mmol/L glycine, and 20% methanol (v/v) for the determination of relative protein content with immunoblot analysis. The transferred membranes were incubated overnight in the blocking buffer, TBST (10 mmol/L Tris–HCl, 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% non-fat milk powder at 4 °C. The membranes were placed at room temperature for 30 min and incubated for 2 h with primary polyclonal antibody against p65 component or phospho-NFκB polyclonal antibody at Ser 536 (1:1000) (Cell Signaling Technology, New England Biolabs, Ont., Canada) in 10 ml blocking buffer with gentle agitation. The membranes were washed three times for 10 min each with 15 ml TBST and then incubated with secondary antibody (1:10,000 goat anti-rabbit IgG horseradish peroxidase conjugate, diluted in TBST containing 1% fat-free milk) at room temperature for 1 h. Antigen-antibody complexes in all membranes were detected by the chemiluminescence ECLplus kit (Amersham–Pharmacia Biotech, Baie d'Urfe, Que., Canada). An Imaging Densitometer (GS-800, Bio-Rad, Mississauga, Ont., Canada) was used to scan the protein band and quantified using the Image Analysis Software Version 1.0. Protein loading was checked in each experiment by staining the membrane with ponceau S staining before immunoblotting [20].

Statistical analysis. The data were expressed as means ± SE. Differences between the control and experimental groups were analyzed by using an unpaired Student's t test. A value of $p < 0.05$ was considered the threshold for statistical significance.

Results

Cardiac function in Ca^{2+}-paradox injury

Five minutes of Ca^{2+}-free perfusion followed by 30 min of Ca^{2+}-repletion caused a dramatic impairment in cardiac performance (Fig. 1). This change was reflected by a 10-fold decrease in LVDP and about a 20-fold increase in LVEDP at 30 min Ca^{2+}-repletion (Fig. 1 and Table 1). A 20-fold decrease in $\pm dP/dt$ in the Ca^{2+}-paradox heart was also detected (Table 1). To determine if PTXF attenuated cardiac dysfunction caused by Ca^{2+}-paradox, hearts were pretreated with PTXF (100 μM) before starting the Ca^{2+}-free perfusion. As depicted in Fig. 1 and Table 1, PTXF treatment significantly improved cardiac function; this was seen by 41% recovery of LVDP, 35% recovery of $\pm dP/dt$, and a significant decrease in LVEDP (this parameter was still 10-fold higher than the control group).

Ca^{2+}-paradox-induced TNF-α production in the heart

As shown in Fig. 2, a dramatic increase in TNF-α level (from 382 ± 25 to 1833 ± 180 pg/g protein) was detected

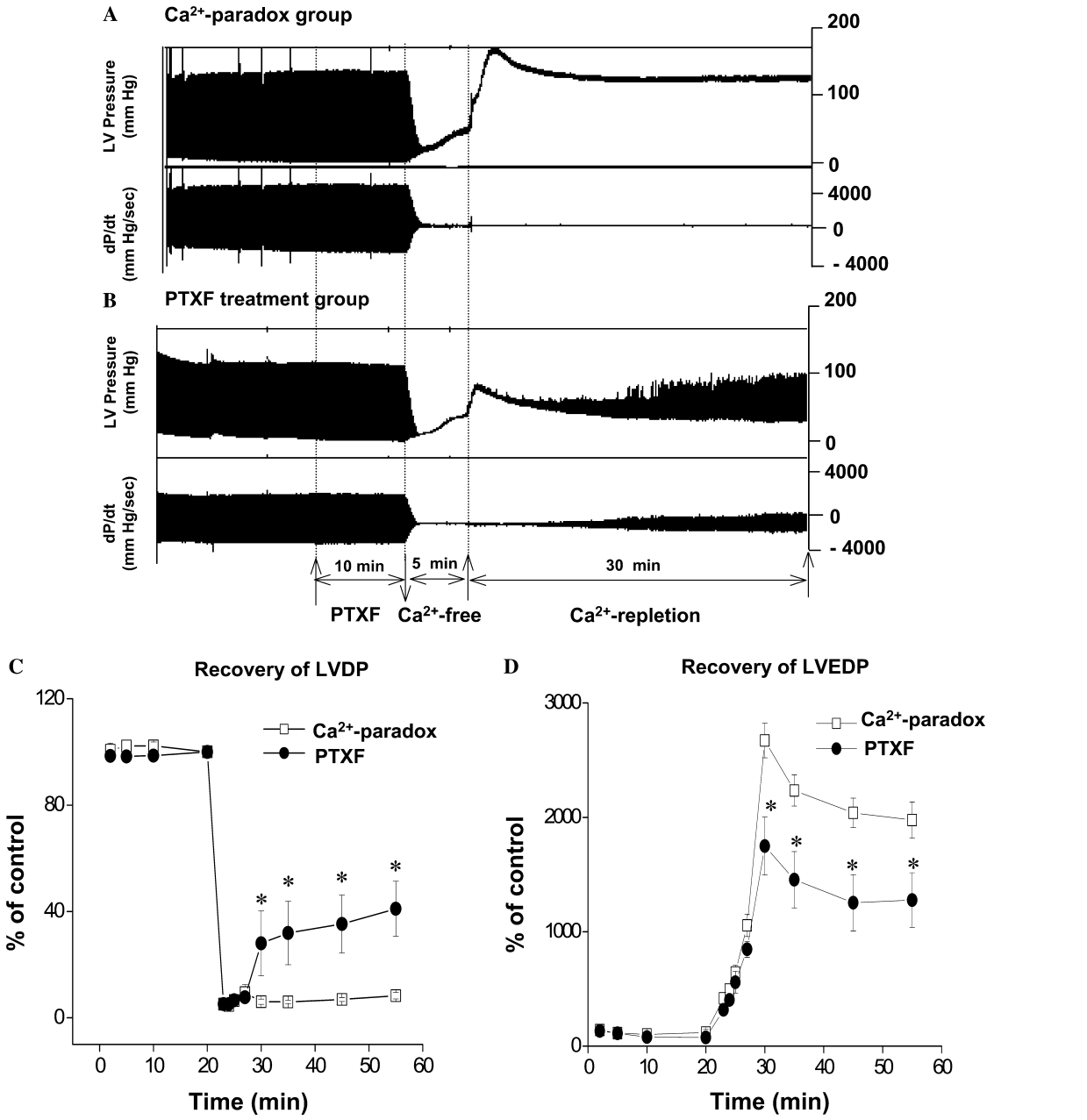

Fig. 1. The tracing represents the recording from heart under Ca^{2+}-paradox without PTXF treatment (A) and with PTXF treatment (B). The effect of PTXF (100 μM) on the alterations of LVDP and LVEDP is shown (C,D) at the different time points during Ca^{2+}-depletion and repletion. Each group consists of six experiments. *$p < 0.05$ vs. Ca^{2+}-paradox group.

in the myocardium after 30 min of Ca^{2+}-repletion, whereas there was no significant change in TNF-α level at 5 min of Ca^{2+}-depletion. However, a significant depression in TNF-α level (784 ± 170 pg/g protein) was detected after 30 min of Ca^{2+}-repletion in the PTXF treatment group (Fig. 2).

Ca^{2+}-paradox-induced alteration in NFκB protein content

In order to investigate the possible mechanism of TNF-α production induced by Ca^{2+}-paradox, protein content of total-NFκB in homogenate, cytosolic, and particulate fractions from control, Ca^{2+}-paradox, and PTXF treated hearts was measured. Fig. 3 shows the representative Western blots of NFκB protein content and analysis of the data. As shown (Fig. 3A), NFκB protein content in the homogenate fraction was significantly reduced (18%) in the Ca^{2+}-paradox group when compared to the control group, unlike the PTXF treatment group. NFκB content in the cytosolic fraction from Ca^{2+}-paradox heart was decreased by 65% (Fig. 3B); PTXF treatment significantly attenuated this

Table 1
Effect of PTXF on cardiac performance of Ca^{2+}-paradox heart

Group	LVDP (mmHg)	LVEDP (mmHg)	$+dP/dt$ (mmHg/s)	$-dP/dt$ (mmHg/s)
Control	111 ± 4.1	3.9 ± 0.5	5938 ± 320	3821 ± 137
Control with PTXF	106 ± 5.5	3.6 ± 0.7	5666 ± 318	3419 ± 193
Ca^{2+}-depletion	$7.3 \pm 1.0^{*}$	$27.5 \pm 2.6^{*}$	$156 \pm 29^{*}$	$150 \pm 32^{*}$
Ca^{2+}-depletion with PTXF	$6.6 \pm 1.2^{*}$	$23.3 \pm 3.1^{*}$	$163 \pm 42^{*}$	$158 \pm 35^{*}$
Ca^{2+}-depletion/repletion	$9.6 \pm 1.5^{*}$	$82.3 \pm 6.0^{*}$	$264 \pm 53^{*}$	$236 \pm 39^{*}$
Ca^{2+}-depletion/repletion with PTXF	$44.3 \pm 11.9^{*,\#}$	$52.5 \pm 12.4^{*,\#}$	$1912 \pm 256^{*,\#}$	$1259 \pm 183^{*,\#}$

Data show the left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), rate of pressure development ($+dP/dt$), and rate of pressure decay ($-dP/dt$).
* $p < 0.05$ ($n = 6$/group) vs. control group.
$^{\#}$ $p < 0.05$ ($n = 6$/group) vs. Ca^{2+}-paradox group.

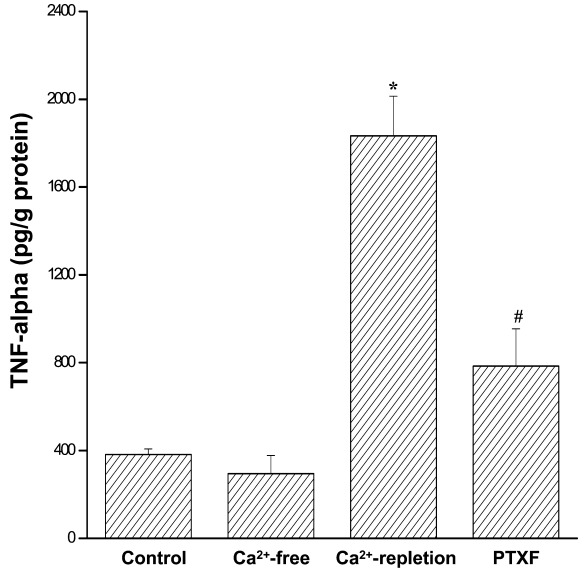

Fig. 2. TNF-α protein level in myocardium subjected to Ca^{2+}-paradox with or without PTXF (100 μM) treatment. Ca^{2+}-free: myocardial TNF-α protein level after 5 min Ca^{2+}-depletion; Ca^{2+}-repletion: myocardial TNF-α protein level after 30 min Ca^{2+}-repletion. Data represent six separate experiments in each group. $^{*}p < 0.05$ vs. the TNF-α level control group, $^{\#}p < 0.05$ vs. Ca^{2+}-paradox group.

change. On the other hand, no significant difference in NFκB content was evident in the particulate fraction from these three groups (Fig. 3C). However, the ratio for NFκB protein in the cytosolic fraction to the homogenate fraction or in the cytosolic fraction to the particulate fraction in the Ca^{2+}-paradox group was lower than the control or the PTXF treatment groups (Figs. 3D and E). This indicates that PTXF prevented the redistribution of NFκB protein induced by Ca^{2+}-paradox.

To examine if NFκB was activated in the Ca^{2+}-paradox heart, the phospho-NFκB content was detected. As shown in Fig. 4A, the level of phospho-NFκB in the homogenate fraction from Ca^{2+}-paradox group was increased by 174% compared to the control group; no significant changes were seen between the PTXF treatment and control groups. While the total NFκB content in homogenate fraction (Fig. 4B) was significantly decreased (15% of control) in the Ca^{2+}-paradox group, there was no change in the PTXF treatment group compared to the control group. The increased ratio of phospho-NFκB to total NFκB (Fig. 4C) indicates the activation of NFκB due to Ca^{2+}-paradox; this ratio was depressed in the PTXF treatment group.

Discussion

In this study, perfusion of briefly Ca^{2+}-depleted hearts with medium containing Ca^{2+} was found to produce cardiac dysfunction associated with a marked increase in TNF-α content. Cardiac dysfunction and increased level of TNF-α have also been reported to occur in hearts subjected to I/R [9,10]. Since TNF-α has been shown to exert negative inotropic effect on the myocardium [2,3], it is possible that the depressed contractile function in Ca^{2+}-paradox and I/R hearts may be mediated through the increased formation of TNF-α. In this regard, it should be noted that TNF-α has been observed to interfere with Ca^{2+} homeostasis, disrupt the excitation–contraction coupling, and desensitize the β-adrenoceptor signal transduction [3,21]. In addition, TNF-α has been shown to increase the production of nitric oxide (NO), and thus decrease the sensitivity of myofilaments to Ca^{2+}, which in turn depresses the contractile function [22,23]. In contrast to the reports showing adverse effects of TNF-α on the heart, different investigators have observed that TNF-α may have cardioprotective effects during I/R [3,24–26]. Nelson et al. [26] have indicated that the pre-treatment with TNF-α, 24 h before I/R period, resulted in improved cardiac contractile function in rabbits. Furthermore, TNF-α knockout mice have been reported to display larger infarct size than normal mice after undergoing coronary ligation [24,25]. Recent studies in our laboratory have suggested that TNF-α at low concentrations is cardioprotective as part of the innate immunity response, whereas at high concentrations this cytokine is a cardiodepressant and mediates cardiac injury [3]. Although the exact mechanisms for the involvement of TNF-α in

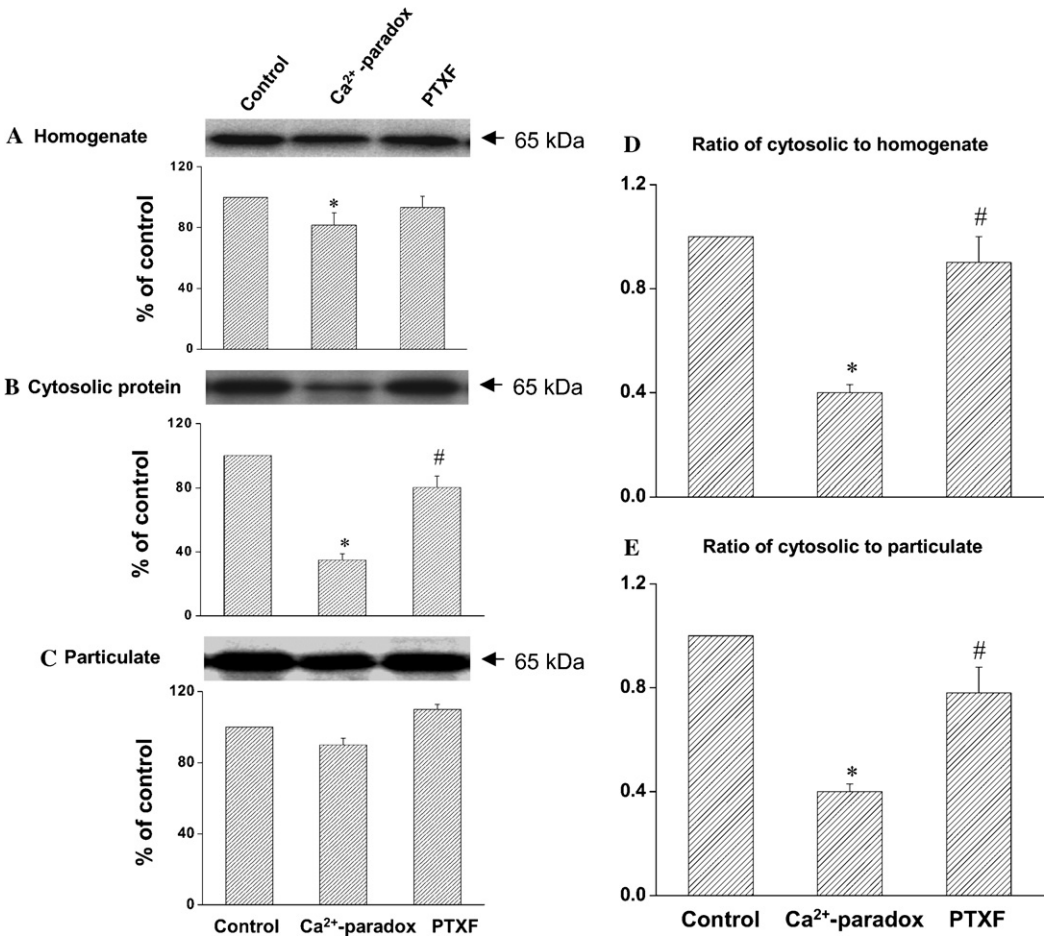

Fig. 3. Western blotting analysis showing the protein level of NFκB in homogenate fraction (A), cytosolic fraction (B), particulate fraction (C), and Ca^{2+}-paradox heart with or without PTXF (100 μM) treatment. The ratio of protein content of NFκB in cytosolic fraction to homogenate fraction and the ratio of protein content of NFκB in particulate fraction to cytosolic fraction has been shown in (D) and (E). $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. Ca^{2+}-paradox group.

cardiac injury are not understood, TNF-α-induced apoptosis in cardiomyocytes has been shown to be mediated by sphingosine and NO [5,27,28]. Thus, our observations showing the elevated level of TNF-α in the Ca^{2+}-paradox hearts and the results from other laboratories [9,10] indicating increased formation of TNF-α in hearts subjected to I/R support the view that an excessive amount of TNF-α may promote cellular injury [11,13,29].

Since NFκB is the key transcription factor that regulates TNF-α gene expression [30], NFκB has been shown to be activated and translocated to the nucleus for the production of TNF-α in the I/R heart [17,31]. The involvement of NFκB in the production of TNF-α in the Ca^{2+}-paradox heart is evident from the fact that the ratio of phospho-NFκB to total NFκB, an index of NFκB activation, was increased whereas the ratio of cytosolic to particulate NFκB, an index of subcellular redistribution, was decreased upon induction of Ca^{2+}-paradox. A slight but significant reduction in total NFκB in the homogenate fraction from Ca^{2+}-paradox heart may be due to leakage of this transcription factor from the myocardium. It is also pointed out that the observed increase in TNF-α in the Ca^{2+}-paradox heart may not reflect the true value indicating the formation of TNF-α due to the leakage of this cytokine during Ca^{2+}-repletion of the Ca^{2+}-depleted hearts. Because the intracellular Ca^{2+}-overload is considered to be a major mechanism for the occurrence of cardiac injury in both Ca^{2+}-paradox and I/R hearts [11,14], it is likely that the increased production of TNF-α in both these conditions is due to the intracellular Ca^{2+}-overload. This view is consistent with the finding that intracellular Ca^{2+}-overload is intimately involved in the activation of NFκB in kidney and lymphocytes [32,33]. Thus, it appears that the increased production of TNF-α due to Ca^{2+}-paradox may be a result of the occurrence of intracellular Ca^{2+}-overload, and subsequent activation and translocation of NFκB in the myocardium. Although oxidative stress has also been shown to be involved in the activation of NFκB following I/R injury [34], its participation in Ca^{2+}-paradox heart cannot be ruled out.

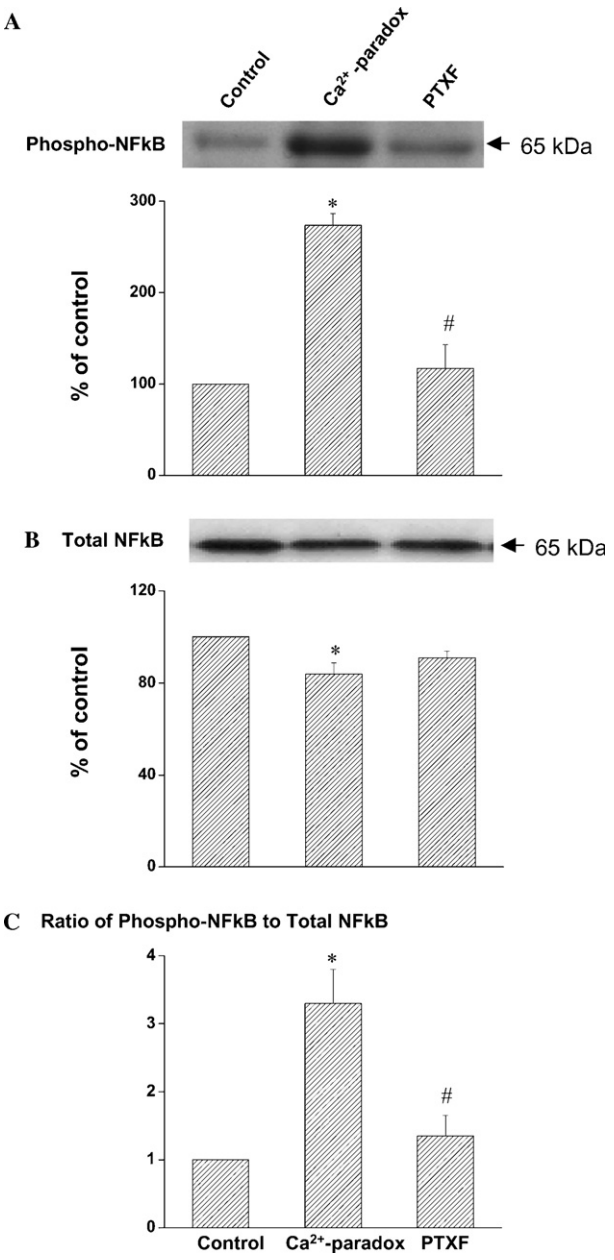

Fig. 4. Western blot analysis showing the protein content of phosphorylated NFκB (phospho-NFκB) (A) and total NFκB (B) in homogenate fraction of Ca^{2+}-paradox heart with or without PTXF (100 μM) treatment. The ratio of protein content of phospho-NFκB to total NFκB in homogenate fraction has been shown in (C). $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. Ca^{2+}-paradox group.

The results in the present study have shown that treatment of the heart with PTXF attenuated the Ca^{2+}-paradox induced increase in LVEDP and improved the recovery of both $+dP/dt$ and $-dP/dt$. Such beneficial effects of PTXF were associated with a reduction in the level of TNF-α as well as depression in the activation and translocation of NFκB in the Ca^{2+}-paradox heart. It should be pointed out that PTXF has been demonstrated to inhibit phosphodiesterase activity, elevate the level of cAMP, activate protein kinase A, and thus reduce the formation of TNF-α [18,35,36]. Furthermore, this agent has been shown to improve cardiac performance by reducing TNF-α level in cardiomyopathic and failing human hearts [15,16]. PTXF has also been considered to produce beneficial effects on the ischemic heart and skeletal muscle due to its potential anti-inflammatory actions [37–39]. Thus, it appears that PTXF may improve cardiac function in Ca^{2+}-paradox heart by reducing the accumulation of TNF-α in the myocardium; however, other mechanisms for the beneficial effects of PTXF in cardiac function cannot be ruled out. Since TNF-α has been reported to block ryanodine receptors as well as decrease the phosphorylation of phospholamban and troponin I in cardiac myocytes [21,40], it appears that lowering of TNF-α level due to PTXF may promote the functional activities of the sarcoplasmic reticulum and myofibrils for improving cardiac performance.

Acknowledgments

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